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Reconstitution and characterization of the human neutrophil *N*-formyl peptide receptor and GTP binding proteins in phospholipid vesicles *

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We have developed a unilamellar phospholipid vesicle system which contains the *N*-formyl peptide receptor and GTP binding proteins. Several detergents were investigated but only two, octyl glucoside (35 mM) and deoxycholate (7 5 mM), were capable of extracting *N*-formyl peptide receptor from neutrophil membranes in a form which remained functionally active upon reconstitution into phospholipid vesicles. Extracted proteins were reconstituted into phosphatidylcholine vesicles by passage over a Sephadex G-50–80 column The reconstituted formyl peptide receptor could bind [^{3}H]FMLP (^{3}H-labeled fMet-Leu-Phe) and [^{125}I]FMLPL-SASD (^{125}I-labeled *N*-formylmethionylleucylphenylalanyl-*N*^{6}-(2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionyl)lysine) while the endogenous G protein could bind [^{35}S]GTPγS. Furthermore, the functional interaction of the two proteins was preserved Addition of the nonhydrolyzable guanine nucleotide, GTPγS, shifted the *N*-formyl peptide receptor from a high- to a low-affinity binding state for ligand. The development of this in vitro reconstitution system should provide a basis to study the mechanism of interaction of the *N*-formyl peptide receptor and the G protein.

Introduction

The *N*-formyl peptide receptor binds chemotactic peptides secreted by foreign pathogens leading to directed migration and stimulation of the microbiocidal functions of human leukocytes The receptor has been partially purified and characterized [1–3] It is an integral membrane glycoprotein of approximately 50–70 kDa Treatment of the receptor with endo-β-*N*-acetylglucosaminadase (endo F) results in the removal of two *N*-linked oligosaccharide chains producing a protein of 33 kDa [4] The receptor has been shown to possess a two-affinity binding state for ligand which appears to be modulated by a G protein (guanine nucleotide binding protein) When the G protein is associated with the receptor, a high-affinity state for the ligand exists Dissociation of the receptor-G protein complex by guanine nucleotides induces the low-affinity binding state [5,6]

Many receptors are coupled with G proteins which act in the signal transduction of hormone binding to the interior of the cell (for recent reviews see Refs 7–9) G proteins are heterotrimers composed of α-, β-, and γ-subunits The β- and γ-subunits are closely associated and may participate in anchoring the subunit to the plasma membrane The α-subunit is responsible for the heterogeneity amongst the various G proteins The neutrophil G proteins are involved in the signal transduction process through which the *N*-formyl peptides stimulate neutrophil function [10] Interaction of chemoattractant with *N*-formyl peptide receptors activate G proteins which appear to activate phospholipase C [11,12] Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) results, yielding diacylglycerol and

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Abbreviations G protein, guanine nucleotide binding regulatory protein, GTPγS, guanosine 5′-(γ-thio)triphosphate, NAD, nicotinamide adenine dinucleotide, FMLP, *N*-formylmethionylleucylphenylalanine fMet Leu-Phe, FMLPL-SASD, *N*-formylmethionylleucylphenylalanyl-*N*^{6}-(2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionyl)lysine, NP40, Nonidet P-40, CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, DFP, diisopropyl fluorophosphate, PMSF, phenylmethylsulfonyl fluoride, CHES, 2 (*N*-cyclohexylamino)ethanesulfonic acid, EDTA, ethylenediaminetetraacetic acid, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid SDS sodium dodecyl sulfate

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inositol 1,4,5-trisphosphate (IP_3). Both products lead to the activation of protein kinase C [13]. Protein kinase C in turn phosphorylates proteins which may elicit the responses characteristic of neutrophil activation including superoxide production and degranulation. The pertussis toxin-sensitive G protein which appears to be associated with the *N*-formyl peptide receptor has been purified and characterized [14,15]. The α-subunit is 40 kDa, it can be ADP-ribosylated by pertussis toxin, and belongs to a family of inhibitory G proteins (G_i). This subunit has been suggested to be $G_i\alpha_2$ and has been cloned from a differentiated HL-60 cell cDNA library [16,17]. Recent reports demonstrate the existence of other neutrophil GTP-binding proteins with molecular weights of 22 000 to 26 000 which are not apparently associated with β- and/or γ subunits [18,19]. These proteins could potentially play a role in the neutrophil signal transduction process. A 26 kDa GTP-binding protein has been suggested to interact with the *N*-formyl peptide receptor [20].

Previous work with the *N*-formyl peptide receptor that had been covalently labeled has demonstrated the ability of a number of detergents to effectively extract the receptor from neutrophil membranes [1–3,21]. In most cases, detergent solubilization was accompanied by the inactivation of the ability of the receptor to bind ligand. While digitonin has been shown to extract and preserve receptor in a form able to bind ligand, this detergent has a number of disadvantages for further purification and reconstitution work. It has been reported for a number of types of receptors that the presence of detergent can inhibit ligand binding to the solubilized receptor [22–24]. Subsequent removal of detergent by reconstitution into appropriate phospholipid vesicles systems can result in recovery of receptor binding activity. Such a reconstitution approach has thus proven extremely useful in purification and study of the receptors of the hormone-sensitive adenylate cyclase system, for example [22–25]. There have been few reports involving reconstitution of an active *N*-formyl peptide receptor function into systems suitable for further investigations. Hoyle and Freer [26] reported the reconstitution of a Nonidet P-40 solubilized and affinity purified receptor from HL-60 into liposomes, but failed to characterize their methods or the reconstituted receptor in any detail. A recent report from Williamson et al. [27] has described the reconstitution of the CHAPS-solubilized receptor from rabbit neutrophils in a functional form.

In order to develop a workable method for extraction, reconstitution, and study of the *N*-formyl peptide receptor, we have taken the approach of optimizing the methodologies in order to obtain fully active receptor. Active receptor is defined as that form able not only to bind the *N*-formylmethionyl peptide ligand with appropriate affinity and specificity, but also to interact functionally with its GTP binding protein signal transduction partner. In this paper, we describe methods for achieving this goal and characterize the reconstituted system in some detail.

Materials

Phosphatidylcholine (1-palmitoyl-2-oleoyl) was purchased from Avanti Polar Lipids Inc., Birmingham, AL. Cholesterol was obtained from Sigma. [^{14}C]Phosphatidylcholine, [^{35}S]GTPγS, ^{32}P-labeled NAD, ^{3}H-labeled FMLP, and [α-^{32}P]GTP were obtained from New England Nuclear. ^{125}I-labeled FMLPL-SASD ligand was prepared according to Allen et al. [28]. The detergents used were octyl glucoside from Calbiochem, deoxycholate, cholate and NP40 from Sigma, digitonin from Gallard-Schlesinger, and CHAPS from Pierce. Low molecular weight protein standards were from Bio-Rad Labs. *Bordetella pertussis* toxin was purchased from List Biological Labs Inc. Protein assay reagents were obtained from Pierce. Other chemicals or reagents were the best available grades.

Methods

Preparation of neutrophil membranes

Human blood was obtained the day of the preparation from voluntary donors. White blood cells were isolated from whole blood as described [29], and the neutrophil content after isolation was determined to be approx. 85–90%. Purified neutrophils were DFP-treated at a final concentration of 2.5 mM, then disrupted by nitrogen cavitation at 4°C in the presence of additional proteinase inhibitors, 2 mM PMSF and 20 μg/ml chymostatin. The membrane fraction was isolated by differential sedimentation through sucrose [30]. Isolated membranes were washed with 25 mM Hepes (pH 7.5), 0.25 M sucrose (HS buffer) and stored at −70°C ready for use.

Receptor and G protein extraction from membranes

Membranes were incubated for 30 min on ice with 1 M NaCl in HS buffer, centrifuged at 100 000 × *g* in a Beckman Ti50 rotor for 45 min at 4°C, washed once with HS buffer, then resuspended at approx. 5 mg/ml in the extraction buffer. The sodium chloride treatment removes approx. 25–35% of the total protein content from the membranes without significantly reducing levels of [^{3}H]FMLP binding. Detergent extractions were performed in a final buffer solution containing 20 mM Hepes (pH 7.5), 1 mM NaEDTA, 0.25 M sucrose, 1 mM DTT, 2 mM PMSF, 20 μg/ml chymostatin, and 250 mM NaCl. The detergent was added last and incubation was for one hour on ice. Precipitable material was centrifuged at 100 000 × *g* in a Beckman Ti50 rotor for 50 min. The supernatant (detergent extract) was

withdrawn and the remaining material was washed once with HS buffer, recentrifuged, and checked for the presence of remaining receptor by [^{3}H]FMLP binding The detergent extract does contain other membrane proteins besides the *N*-formyl peptide receptor and G proteins However, these additional proteins do not interfere with the reconstitution or ligand binding

Phospholipid-micelle formation

Phosphatidylcholine (PC) and cholesterol, solubilized in ethanol, were combined to give a final concentration upon aqueous resuspension of 10 5 mg/ml and 1 05 mg/ml, respectively The organic solvent was evaporated with nitrogen and the lipid was resuspended in buffer containing 25 mM Hepes (pH 7 5), 1 mM NaEDTA, 250 mM NaCl, 2 mM $MgCl_2$ with detergent added at a concentration equal to that used for the receptor extraction from the membrane The micelles were incubated at room temperature for 30 min and stored on ice until use

Phospholipid vesicle generation

The detergent extract was added drop-wise to the preformed phospholipid micelles at a lipid/protein ratio of approx 5 1 (w/w), and incubated on ice for 30 min The mixture was chromatographed over a Sephadex G-50–80 column, 0 8 cm × 21 cm [31] The void volume, predetermined with Blue dextran, and the first fraction following the void volume were collected and centrifuged separately at 100 000 × *g* in a Ti50 rotor for 3 h to concentrate the unilamellar vesicles The vesicles were resuspended in the detergent extraction buffer without the detergent

Receptor binding and G protein assays

Membranes or vesicles were added to a buffer solution containing a final concentration of 200 mM sodium phosphate (pH 6 8), 2 mM $MgCl_2$, and 0 9 mM $CaCl_2$ [32] The binding activity of the receptor was assayed at a saturating concentration of 120 nM [^{3}H]FMLP (1 33 10^5 cpm/pmol) The samples, assayed in duplicate, were incubated at room temperature for 40 min Nonspecific samples had a 1000-fold excess of unlabeled FMLP ligand added The sensitivity of binding to guanine nucleotide was assayed similarly, but in the presence of $5 \cdot 10^5$ M GTPγS. Samples were filtered over Millipore GF/B glass filters and washed four times with 2 ml of cold 25 mM KH_2PO_4 (pH 6 8), 1 mM NaEDTA, 100 mM KCl, and 2 mM $MgCl_2$ Whatman GF/F filters were also tested and gave the same results as the Whatman GF/B, data not shown The filters were dried and counted in a liquid scintillation counter Binding of [^{125}I]FMLPL-SASD was performed at 5 nM for 30 min on ice, and subsequently exposed to an ultraviolet light source to covalently crosslink the ligand [28] The buffer used for this ligand binding was changed to 200 mM CHES (pH 9 0), 2 mM $MgCl_2$, and 0 9 mM $CaCl_2$ SDS-polyacrylamide gel electrophoresis [33] of this receptor-ligand complex in membranes and vesicles was used to visually detect and quantify the presence of the receptor after autoradiography

The binding of [^{35}S]GTPγS was assayed as described previously [34] ADP-ribosylation was performed with pertussis toxin and [^{32}P]NAD according to Bokoch et al [35] Detection of the low-molecular-weight G proteins by the binding of [α-^{32}P]GTP was based on Bhullar and Haslam [36] Two changes were made in the procedure The specific activity of the [α-^{32}P]GTP used was reduced to 800 Ci/mmol and the protein filters were preblocked with 1% dry milk Western blotting was performed essentially as described by Towbin et al [37] Antibody R16, 17 was utilized at a final dilution of 1 200, the characterization of this $G_i\alpha$-subunit antibody is described in Ref 38

Electron microscopy

Vesicles were negatively stained with uranyl formate and viewed under an electron microscope to achieve an approximation of their size and detect their unilamellar nature [39]

Results

Detergent extraction of N-formyl peptide receptor

Detergent extractions of neutrophil membranes were performed using a concentration of detergent slightly above the critical micelle concentration, in order to minimize the use of unnecessarily high detergent concentrations which may potentially denature the membrane proteins during the extraction [40] Several different detergent concentrations were investigated until a maximum amount of receptor was extracted, as determined by [^{3}H]FMLP binding to the post-extraction pellet The optimum detergent concentrations for various detergents were determined and are listed in Table 1 There is a difficulty inherent in the above determinations The [^{3}H]FMLP may not bind to receptor still present in the membrane which has been denatured or 'masked' by exposure to detergent Thus can result in an overestimation of the percentage of actual receptor extracted This problem was addressed by using [^{125}I]FMLPL-SASD to label membranes prior to extraction The autoradiograms of the detergent extracted receptor (supernatants) and nonextracted receptor (pellets) are shown in Fig 1 The autoradiograms clearly illustrate deoxycholate and octyl glucoside as the detergents which extract the maximum amount of receptor This is indicated by the large amount of covalently labeled receptor detected in the supernatant, lanes 2S and 6S, with minimum amounts of receptor remaining in the pellet, lanes 2P and 6P Quantitation of all lanes by laser scanning densitometry allowed the determina-

tion of the percentage of detergent-extracted receptors listed in Table I Only deoxycholate and octyl glucoside extracted the same amount of receptor when assessed by both binding methods The remaining detergents show significant discrepancies between the two techniques This result suggests that these latter detergents either cause denaturation of *N*-formyl peptide receptor within the membrane or are able to prevent binding of ligand to the remaining membrane associated receptor and may therefore be less suitable for obtaining active receptor

The percentage of G protein extracted by the various detergents is also listed in Table I Deoxycholate and octyl glucoside extracts contain the greatest amount of G protein Although cholate and CHAPS also have a comparable amount of G protein in the extract, they are less effective in extracting receptor

Receptor incorporation into phospholipid vesicles

The vesicle generation process assessed using deoxycholate-extracted receptor, prelabeled with [^{125}I]FMLPL-SASD, is shown in Fig 2. The initial membranes, lane 1, show the presence of the *N*-formyl peptide receptor which migrates on the SDS-polyacrylamide gel with an apparent molecular mass of 50–70 kDa The 1 M NaCl wash of the membranes, lanes 3, 4, shows no significant loss of the receptor by this procedure The HS buffer rinses, lanes 5 and 7, also show an absence of receptor Most of the receptor from lane 4 was extracted by deoxycholate as seen in lane 6 The receptor remaining in the pellet after deoxycholate extraction, lane 8, contains approx 10-times the amount of gel loaded protein as lane 6, yet an almost equal band intensity Quantitation by laser scanning densitometry indicates that approx 12% of receptor remains in the pellet and thus, 88% of the receptor is extracted from the membranes in this experiment. The extract was subsequently combined with PC micelles, lane 9, and chromatographed on a Sephadex G-50

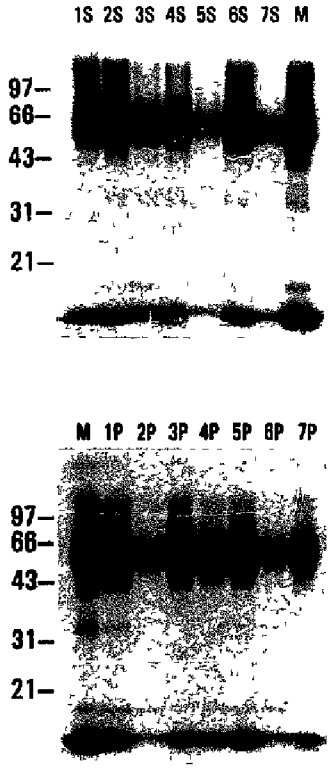

Fig 1 Detergent extractions of neutrophil membranes labeled with [^{125}I]FMLPL-SASD, as described in Methods (1) cholate, 20 mM, (2) deoxycholate, 7 5 mM, (3) digitonin 1 0% (4) NP40 3 0 mM (5) CHAPS, 15 mM, (6) octyl glucoside, 35 mM, and (7) digitonin, 0 5% Supernatants (S) and pellets (P) are shown on separate 11% SDS-polyacrylamide gels with starting membranes (M) The *N*-formyl peptide receptor and its photoaffinity ligand are detected as the broad radiolabeled band between 50000 and 70000 [28]

TABLE I

Percentage of N-formylpeptide receptor and G protein extracted from neutrophil membranes

100% represents what is initially present in the starting membranes for both proteins

Detergent		Percentage extracted		
		[^{3}H]-FMLP	[^{125}I]-FMLPL	[^{35}S]-GTPγS
1 Deoxycholate	7 5 mM	96	95	89
2 Octyl glucoside	35 mM	96	94	77
3 NP 40	3 0 mM	90	79	48
4 Cholate	20 mM	60	51	79
5 Digitonin	1 0%	80	46	45
6 Digitonin	0 5%	78	30	25
7 CHAPS	15 mM	75	24	71

column Radioactive counts were recovered in the void volume, (V_0), the two succeeding fractions after the V_0, as well as in the elution volume, (the third and subsequent fractions after the V_0) The elution volume, lanes 10 and 11, show the absence of receptor and represent free ligand The V_0, lane 12, shows the reconstituted receptor, which elutes with the phospholipid and GTP binding protein The first detectable loss in the reconstitution process occurs here, with approx 50% of the initial receptor not being recovered after passage over the column, although the column is preblocked with bovine serum albumin and eluted with 250 mM NaCl in the buffer Lanes 13 and 14 contain the two succeeding fractions after the V_0, respectively, and illustrate the

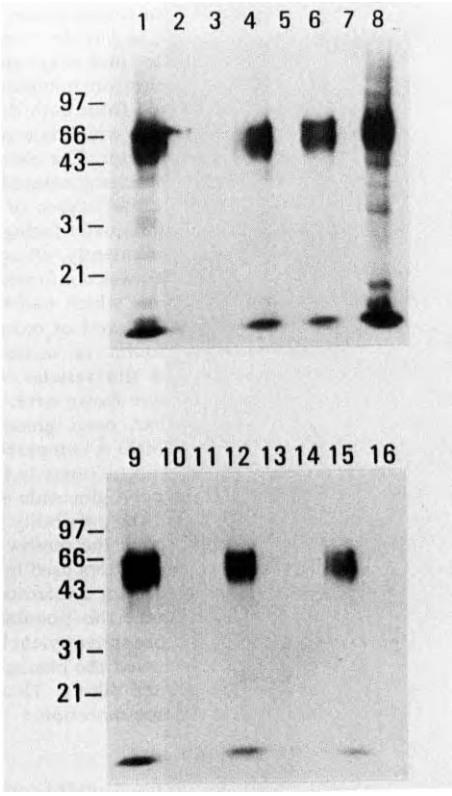

Fig 2 The steps in phospholipid vesicle production were assessed by following [^{125}I]FMLPL-SASD covalently labeled receptor in the starting membranes, as described in Methods Approx 17 μg of membrane protein, based on starting membrane protein concentration is loaded per lane An 11% SDS-polyacrylamide gel was used Lanes (1) initial membranes after −70 °C thaw and labelling, (2) supernatant after initial membrane centrifugation to pellet (3) supernatant after 1 M NaCl wash, (4) membranes after 1 M NaCl wash (5) supernatant after HS buffer rinse, (6) deoxycholate detergent extraction (7 5 mM) of membranes, (7) supernatant of HS buffer pellet rinse after detergent extraction, (8) pellet remaining after detergent extraction (15) μg protein loaded instead of 17 μg), (9) detergent extract plus PC micelle sample loaded on a Sephadex G-50–80 column, (10 and 11) elution volume fractions third and subsequent fractions after the void volume, containing non-covalently attached [^{125}I]FMLPL-SASD counts, (12) void volume containing vesicles, (13 and 14) first and second fractions after the void volume, (15) void volume centrifuged to concentrate vesicles, (16) first and second fractions after the void volume combined and centrifuged

absence of receptor. Lastly, after centrifugation to concentrate the vesicles, minimal loss of receptor is seen for V_0, lane 15 (80–85% recovery of phospholipid, as determined with [^{3}H]phosphatidylcholine (not shown)), and still no detectable receptor is present in the two fractions following V_0 lane 16

Comparison of various detergents for reconstitution of active receptor

The efficacy of other detergents for reconstitution into phospholipid vesicles were further investigated based on the data derived from Table I Octyl glucoside CHAPS, and NP40 were compared to deoxycholate The CHAPS and NP40 extracts produced no detectable reconstitution based on [^{3}H]FMLP ligand binding. Both deoxycholate and octyl glucoside did allow reconstitution of active *N*-formyl peptide receptor which bound [^{3}H]FMLP The octyl glucoside at 35 mM produces the highest degree of receptor reconstitution ranging from 10 to 30% of the initial receptor with a typical value of approx 22% Deoxycholate at 7 5 mM was not as effective in allowing receptor reconstitution, with only 4–8% of the receptor reconstituted and a 7% typical value Nonspecific [^{3}H]FMLP binding in both detergents was approx 5–10% of total binding and was subtracted from total binding to determine the percent of reconstitution

The percentage of deoxycholate-extracted receptor and octyl glucoside-extracted receptor reconstituted into

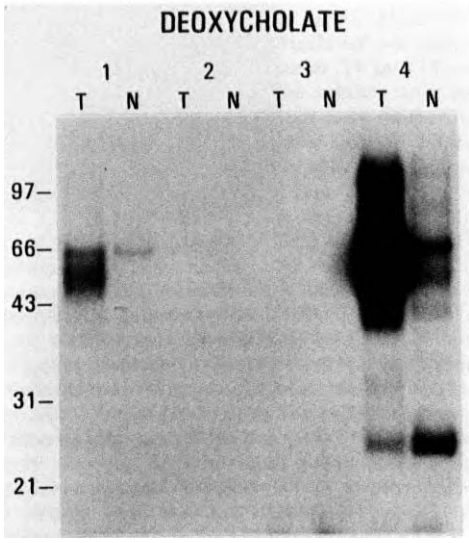

Fig. 3 Deoxycholate extraction of the *N*-formyl peptide receptor from neutrophil membranes and reconstitution into phospholipid vesicles Prior to electrophoresis, [^{125}I]FMLPL SASD was covalently bound to all samples, as described in Methods Samples are (1) V_0 fraction, (2) V_{0+1} fraction, (3) phospholipid micelles and (4) membranes T represents total binding and N represents nonspecific binding Approx 10 μg of protein are loaded per lane on an 11% SDS-polyacrylamide gel

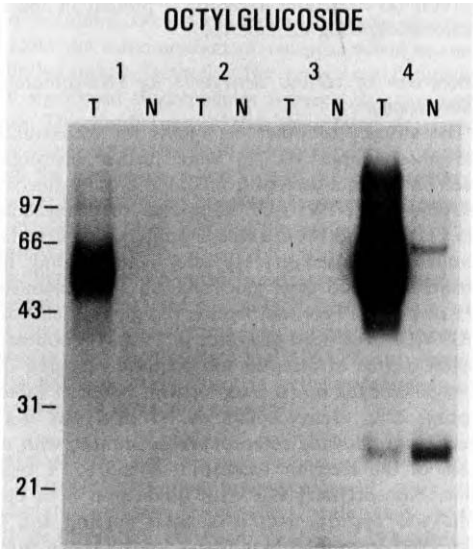

Fig 4 Octyl glucoside extraction of the *N*-formyl peptide receptor from neutrophil membranes and reconstitution into phospholipid vesicles Methods and samples are as described for Fig. 3

phospholipid vesicles was also assessed using [^{125}I]FMLPL-SASD and shown in Figs 3 and 4 The receptor can be clearly visualized for both detergents, lanes 1T and 4T, demonstrating incorporation of radiolabel into vesicles and membranes, respectively The first fraction after the void volume, lane 2T, and phospholipid micelles alone, lane 3T, have no detectable receptor A 66 kDa nonspecific binding protein is present in lanes 1 and 4 This could be bovine serum albumin, which can bind [^{125}I]FMLPL-SASD and is utilized in various methods described here In addition, lane 4 contains a 24 kDa protein which may represent a degradation product of the receptor [41] An anomalous increase of [^{125}I]FMLPL-SASD binding in the presence of unlabeled ligand can also be seen for this protein. Quantitation of the receptor by laser scanning compared the amount of active receptor reconstituted into the vesicles to the initial amount present in the membrane Similar to the [^{3}H]FMLP data, this amount was less for deoxycholate than for octyl glucoside For deoxycholate 6 9% of the receptor was reconstituted by assay with [^{125}I]FMLPL-SASD and 7 0% with [^{3}H]FMLP On the other hand, for octyl glucoside the [^{125}I]FMLPL-SASD detected 18% reconstituted receptor compared to 31% for [^{3}H]FMLP The two assay techniques thus demonstrated that the *N*-formyl peptide receptor can be reconstituted with retention of ligand binding activity into vesicles using both detergents Furthermore, the efficacy of octyl glucoside is approx 3-fold that of deoxycholate

Physical properties of the phospholipid vesicle system

The structure of the phospholipid vesicles formed upon reconstitution of deoxycholate- and octyl glucoside-extracts was investigated by electron microscopy Fig 5 shows that the vesicles formed from both detergent extracts are unilamellar in nature which is evident by the bright ring around a dark interior The electron micrographs also indicate that the vesicles generated are not sealed These vesicles should allow access of the ligand to receptors which may be potentially facing the inside rather than the exterior Consequently, all active receptor present can be detected This was confirmed by performing binding assays on vesicles which had been permeabilized with 0 005% digitonin, levels of receptor detected were similar to those found in untreated vesicles The approximate sizes of the vesicles were measured from other micrographs not shown here Deoxycholate vesicles were larger than octyl glucoside vesicles having a range of 120 Å to 3000 Å compared to 380 Å to 1200 Å Average values of approximately 1100 Å for deoxycholate and 600 Å for octyl glucoside were determined This size estimate raised the possibility that the smaller vesicles might pass through the meshwork-type pores of the Millipore glass fiber filters used in the [^{3}H]FMLP binding assay, resulting in an underestimation of the actual binding To examine this possibility, vesicles were produced with [^{14}C]phosphatidylcholine Filtration of the labeled vesicles allowed the passage of only 15–20% of vesicles through the filters Thus, a minimal amount of the vesicles escape detection

Presence of GTP binding proteins in reconstituted vesicles

The presence of G proteins in the reconstituted phospholipid vesicles was determined by several methods Fig 6 shows that the vesicles contain a *B pertussis* toxin substrate which has a molecular mass of 40 kDa similar to that found in the membranes This 40 kDa G protein α-subunit was also detected by antibody R16, 17 which reacts with 'G_i' α-subunits, as illustrated in the Western blot, Fig 7 This protein is likely to represent the major *B pertussis* toxin substrate (G_n) of neutrophils, which is thought to interact with the *N*-formyl peptide receptor [14,15,38] The presence of G_n in the vesicles further is verified by the effect of GTPγS Addition of GTPγS to the [^{3}H]FMLP binding assay produces a large decrease in the ligand binding. For instance, the percentage of reconstituted [^{3}H]FMLP binding receptor by deoxycholate extraction was 8% This was decreased to 0 3% in the presence of GTPγS Similarly, for the octyl glucoside-extract [^{3}H]FMLP binding of the reconstituted receptor was 31% which was decreased to 0 8% in the presence of GTPγS Thus, resembling the effect in the membranes, GTPγS uncouples the G protein from the receptor shifting it to a low-affinity binding state for ligand

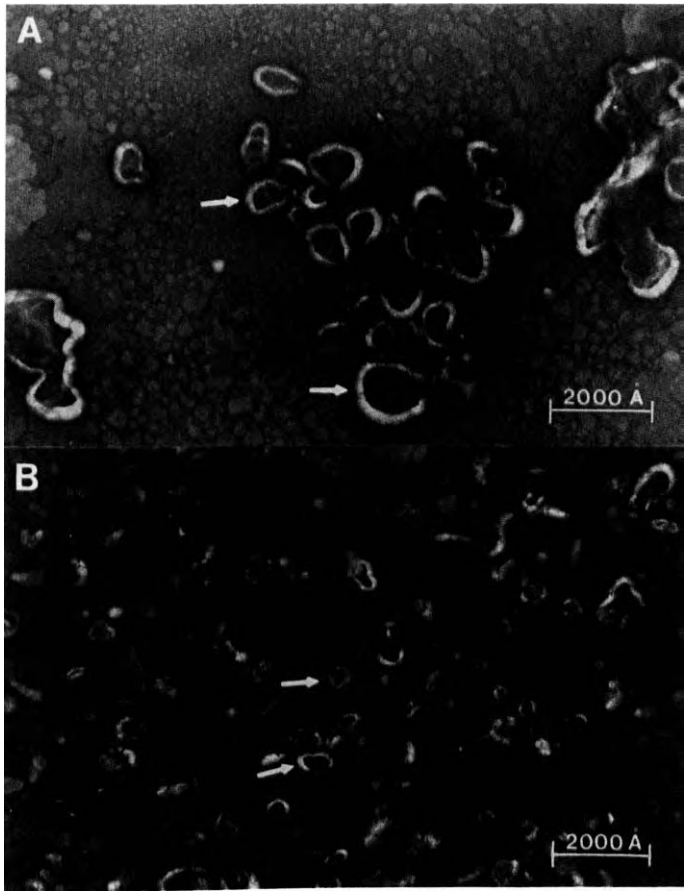

Fig. 5 Electron micrographs of phosphatidylcholine vesicles negatively stained with uranyl formate (A) Vesicles prepared with deoxycholate extracts (7 5 mM) of neutrophil membranes and (B) vesicles prepared with octyl glucoside extracts (35 mM)

In addition to G_n, low-molecular-weight GTP binding proteins were detected in reconstituted vesicle preparations prepared using octyl glucoside- or deoxycholate-extracted membranes Fig. 8 shows the presence of a [α-^{32}P]GTP-labeled doublet consisting of the 24 kDa and the 26 kDa proteins as previously reported [18]

Functional interaction of receptor with G protein(s) in the reconstituted system

A binding analysis of the *N*-formyl peptide receptor in vesicles and membranes, Fig 9, was performed to further characterize the properties of the reconstituted receptor The binding isotherms indicate that the receptor has similar equilibrium dissociation constants in the native membrane ($K_d \simeq 2$ nM) and in the reconstituted vesicle ($K_d \simeq 10$ nM) The decreased binding affinity of the receptor reconstituted into the vesicles is possibly a result of exposure of the receptor to detergent and removal from the membrane environment to an artificial phospholipid bilayer

It is probable that the binding we measure represents the high-affinity ligand-receptor-G protein ternary complex since we are unlikely to detect the low-affinity state of the receptor representing the form uncoupled from the G protein with the filtration assay utilized [5,6,10] This is indicated by the ability of GTPγS to inhibit assayable [^{3}H]FMLP binding to the reconstituted receptor The specific binding of [^{3}H]FMLP assayed in deoxycholate-extracted receptor reconstituted vesicles was reduced by 96%, and that in octyl glucoside reconstituted vesicles by 97% in the presence of 5 10^{-5} M GTPγS Thus reduction in binding was specific for guanine nucleotide Thus, as in membranes, the recon-

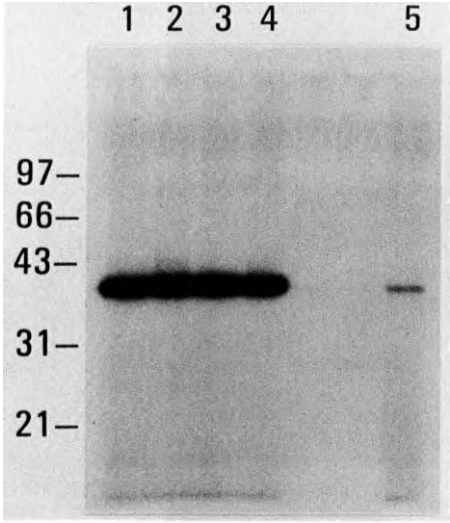

Fig 6 ADP-ribosylation of phospholipid vesicles from several different reconstitutions using 7 5 mM deoxycholate-extracted membranes (Lanes 1–4) at 20 μg membrane protein per lane, and neutrophil membranes (Lane 5) at 2 μg in the presence of *B pertussis* toxin The SDS-polyacrylamide gel was 11%

stituted receptor is able to functionally interact with G protein to form the high-affinity, guanine nucleotide sensitive ternary complex

Discussion

We have developed a method for extraction and reconstitution of functional *N*-formyl peptide receptor

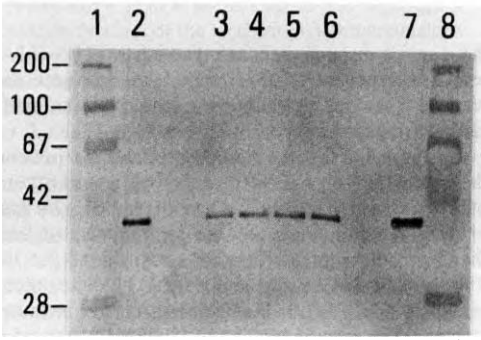

Fig 7 Western blot of neutrophil membranes at 10 μg (2), phospholipid vesicles generated from a deoxycholate extract at approx 32 μg and 36 μg, respectively (3 and 5), and octyl glucoside extract at approx 32 μg and 36 μg, respectively (4 and 6), and purified bovine brain G_i (7) at approx 1 μg Protein standards (1 and 8) are marked on the gel An 11% SDS-polyacrylamide gel was used

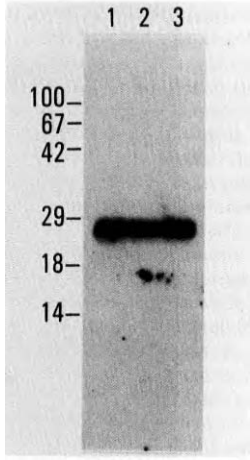

Fig 8 Autoradiogram showing the presence of low molecular G proteins in neutrophil membranes (1), vesicles prepared with 7 5 mM deoxycholate-extracts (2), and vesicles prepared with 35 mM octyl glucoside-extracts (3) A 15% SDS-polyacrylamide gel was loaded with approx 15 μg membrane protein in lane 1, and 30 μg of protein in lanes 2 and 3 The gel was Western blotted and probed with [α-^{32}P]-GTP

into unilamellar phospholipid vesicles We have utilized the criteria of maintaining both ligand binding capability of appropriate affinity, as well as ability to interact with endogenous GTP binding protein(s) as indices of receptor integrity Utilizing both functional assays and

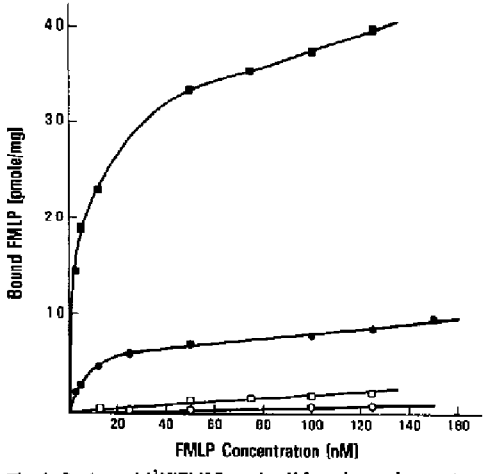

Fig 9 Binding of [^{3}H]FMLP to the *N*-formyl peptide receptor in neutrophil membranes, (total, ■, and nonspecific, □), and in octyl glycoside extract reconstituted phospholipid vesicles, (total, ●, and nonspecific ○)

methods to physically detect receptor and G protein, we have verified the presence of these components throughout the reconstitution procedure and have determined recoveries of receptor at each step

While a number of detergents were tested for their ability to extract receptor and allow recovery of receptor function upon reconstitution, only deoxycholate and octyl glucoside proved reasonably effective Williamson et al [27] have solubilized and reconstituted the receptor from rabbit neutrophils using 1% (15 mM) CHAPS, although receptor recovery was not reported As shown, we had little success with this detergent on the extraction and reconstitution of the *N*-formyl peptide receptor from human neutrophils The rabbit receptor was present at 20 pmol/mg of membrane protein while for the human receptor we measured a concentration of the order of 3–4 pmol/mg of membrane protein Potentially, the higher concentration of receptor allows for its stabilization and reconstitution from the rabbit neutrophils In addition, Marasco et al [2] have extracted the *N*-formyl peptide receptor from rabbit neutrophils using 10 mM CHAPS They report only a 30–70% solubilization of the initial receptor present comparable to our value of 24% for 15 mM CHAPS In this study, octyl glucoside gave optimal results, allowing the reconstitution of approximately 3-fold more active receptor than did deoxycholate Use of octyl glucoside allowed a recovery in the range of 10% to 30% of the receptor initially present in the neutrophil membranes in the final vesicle preparation, as assessed both by [^{3}H]FMLP binding and [^{125}I]FMLPL-SASD labeling. Correction for receptor losses during vesicle concentration and during filtration assays would increase the actual recoveries by 20 to 40% This correction for octyl glucoside extraction produces a receptor recovery range of 12% to 42% with a typical value of 31% This compares with recoveries of 30 to 80% reported for various other receptors reconstituted from crude extracts with comparable methodologies [23,24,42]

The majority of the receptor loss appeared to occur during the gel filtration step utilized for phospholipid vesicle formation Approximately a 50% loss of applied receptor occurred during this procedure, even though the columns were preequilibrated with 1% bovine serum albumin and eluted in the presence of 250 mM NaCl While this loss may be largely due to nonspecific binding of receptor to a Sephadex column, it may additionally represent loss or inactivation of receptor during the detergent removal-vesicle formation process In addition, uncoupling of receptor from G protein may occur during the reconstitution process This would permit incorporation of the receptor into phospholipid vesicles not containing G protein, causing an underestimation of the amount of actual receptor recovered after reconstitution, as assessed by [^{3}H]FMLP binding assay

The reconstituted *N*-formyl peptide receptor retains binding affinity comparable to that of the native receptor It also shows the ability to functionally interact with G proteins present in the vesicles It is not clear at this time whether this R-G coupling actually occurs subsequent to reconstitution into the vesicles or whether the R-G complexes preformed in the native membranes are maintained throughout the reconstitution procedure However, the probability of a vesicle containing both the *N*-formyl-peptide receptor and G protein, if these proteins were not coupled throughout the reconstitution would be 1 in every 250 vesicles for deoxycholate and 1 in every 600 vesicles for octyl glucoside These low probabilities would not predict the percentage reconstitution determined with the [^{3}H]FMLP binding assay The calculations are based on the concentration of *N*-formyl peptide receptor and G protein determined in the detergent extract which can be reconstituted, in addition to the amount of phospholipid used and the vesicle size determined by electron microscopy [43] We have also assumed that there are no interactions between the G protein or the *N*-formyl peptide receptor which may be located in different phospholipid vesicles

In summary, an in vitro reconstitution system has been developed which will allow the study of the *N*-formyl peptide receptor and G proteins Reconstitution of various subunits of the endogenous G proteins in addition to other G proteins will provide insight into G protein–*N*-formyl peptide receptor interactions In addition, future reconstitutions of receptor, G proteins, phospholipase C or other effectors will permit biochemical analysis of the interactions of these substituents and their role in the overall process of neutrophil stimulation

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